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(54) An oxido reductase enzyme system obtainable from P. chrysogenum, the set of genes encoding the same and the use of oxido reductase enzyme systems or genes encoding the same for increasing antibiotic production.

(57) A new oxido reductase enzyme activity obtainable from P. chrysogenum, involved in the production of β-lactams, the set of genes encoding said enzyme activity, and a method to enhance said production by using the above-mentioned oxido reductase system and other oxido reductase systems or genes encoding for the same have been disclosed.

EP 0 462 674 A1

The present invention relates to an enzyme system involved in the production of β-lactam compounds and to the use of enzyme systems for increasing antibiotic production.

Penicillins and cephalosporins are the most widely used antibacterial agents. Penicillins and cephalosporins are secondary metabolites which are industrially produced by filamentous fungi like Penicillium chrysogenum and Acremonium chrysogenum, respectively, in several enzymatic steps (J.R. Miller and T.D. Ingolia, Mol. Microbiol. (1989) 3:689-695).

The main steps in the biosynthetic pathways leading to cephalosporins and penicillins have been elucidated in the past 30 years. The pathways share two enzymatic steps. In the first step a tripeptide is formed from α-aminoadipic acid, cysteine and valine. The enzyme which is responsible for this step is δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase. In the second step the ACV is cyclised by the action of isopenicillin N synthetase (hereinafter referred to as IPNS) or cyclase. The reaction product is isopenicillin N, a compound which contains the typical β-lactam ring structure and which possesses antibacterial activity. The biosynthesis of penicillin involves a unique third and last step in which the α-aminoadipic acid side-chain of isopenicillin N is exchanged for a hydrophobic side-chain. The hydrophobic side-chains commonly used in industrial production are phenylacetic acid (PAA) and phenoxyacetic acid (POA) yielding penicillin G and penicillin V, respectively. The side-chain exchange has been proposed to be a reaction catalyzed by a single enzyme referred to as acyltransferase (AT). Cephalosporins are formed from isopenicillin N in a number of steps including epimerization of isopenicillin N to penicillin N, ring expansion and hydroxylation.

The biosynthetic pathways to penicillins and cephalosporins have almost completely been elucidated and most of the enzymes involved in the biosynthesis towards penicillins and cephalosporins have been purified and characterized (Ingolia et al., Med. Res. Rev., (1989) 9:245-256). The genes encoding these enzymes have been cloned and it is commonly accepted that the introduction of expression vectors containing DNA encoding these enzymes into production strains may improve the yield of β-lactam compounds. Recently, an example of the successful application of the expression of extra copies of genes has been described (Skatrud et al., Biotechnology, (1989) 7:477-485).

It has been recognized that maintaining reducing conditions is important for producing β-lactam compounds. For instance, under non-reducing conditions the tripeptide ACV dimerizes into ACV disulphide (bis-ACV) or possibly into mixed disulphides with other thiol-containing compounds. These disulphides are not used as substrates by IPNS. Furthermore, the enzyme IPNS is less active in non-reducing conditions (Perry et al, Biochem. J. (1988) 255:345-351). Reducing compounds such as dithiothreitol (DTT) are used to maintain reducing conditions in vitro. In this way a process for producing cephalosporins from tripeptide precursors as well as unnatural penicillin and cephalosporin derivatives using isolated enzymes has been developed (US patent 4,510,246 and 4,536,476). Moreover, a process for producing tripeptides using the enzyme ACVS has been described in EP-A-280051. At this moment it is not known how enzymes and disulphide compounds, for instance ACV, involved in the production of β-lactam compounds are maintained in a reduced state in P. chrysogenum or other β-lactam producing microorganisms. Surprisingly a yet unreported enzyme system with reducing properties which is able to reduce bis-ACV amongst other disulphide compounds, has been isolated. The enzyme system comprises two polypeptide components, a high and a low molecular weight polypeptide, and an electron carrier, such as nicotinamide adenine dinucleotide phosphate (NADPH), in its reduced form. The polypeptide components share similarity with thioredoxin reductase and thioredoxin, respectively. It has not previously been mentioned that the thioredoxin system might be involved in the β-lactam production.

Therefore, the present invention provides an oxido reductase system, viz. a thioredoxin reductase system which is obtainable from P. chrysogenum, which is able to reduce ACV disulphide amongst other compounds is provided. This oxido reductase system consists of a high molecular weight (HMW) polypeptide with a molecular weight of about 36 kDa as determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), and a low molecular weight (LMW) polypeptide with a molecular weight of about 12 kDa as determined by SDS-PAGE, and a reduced electron carrier. The high and the low molecular weight polypeptides have been isolated by a combination of gel chromatography, ion-exchange chromatography and affinity chromatography. The HMW component is similar to the thioredoxin reductase obtained from Escherichia coli. The LMW component is similar to known thioredoxin, and possesses similar activities including protein disulphide exchange. Also provided for are the genes encoding the polypeptide compounds of the new oxido reductase system obtainable from P. chrysogenum.

Furthermore, the oxido reductase system of P. chrysogenum has been found to be involved in maintaining enzymes and disulphide compounds, for instance ACV, involved in β-lactam biosynthesis in a reduced state. Also other thioredoxin systems have been found to be applicable for this object. In this way these systems are applicable in the in vitro production of agents, for instance in the production of β-lactam

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antibiotics. Also, genes encoding both the polypeptide components of the new oxido reductase system obtainable from P. chrysogenum and those of the (other) thioredoxin systems can be used in a method to increase the production of metabolites, for instance β-lactam compounds, by growing a microorganism culture consisting of transformants of said microbial host comprising DNA sequences encoding first and second components or functional portions thereof of a thioredoxin system, each DNA sequence under the regulatory control of transcriptional and translational regulatory signals functional in said cells, whereby said components of said enzyme system are expressed and said β-lactam compound is produced; and isolating said β-lactam compound. The present invention further provides DNA constructs comprising at least one gene encoding a polypeptide compound of the new oxido reductase system and a host comprising said construct. Finally, a method for reducing disulphide compounds and compounds involved in the production of β-lactam compounds in vitro has been provided for by contacting, in the presence of a reduced electron carrier, the compounds to be reduced with a composition comprising an oxido reductase system, consisting of a HMW polypeptide and a LMW polypeptide, and being capable of reducing disulphide compounds, or with a LMW polypeptide in the presence of a reducing agent as for instance DTT. Preferably, ACV is reduced in vitro in this way.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Schematic representation of a partial restriction map of the P. chrysogenum derived DNA contained in pPCH-1. The position of the 5'-end of the gene and the direction of transcription of the HMW gene is indicated (arrow).

Figure 2: Partial nucleotide sequence of the P. chrysogenum HMW gene. The open reading frame encoding the N-terminal HMW amino acid sequence is indicated (underlined). The position of an intron is also indicated (undercast).

BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

Sequence listing No. 1 shows part of the nucleotide sequence and deduced amino acid sequence of the P. chrysogenum HMW polypeptide.

Sequence listing No. 2 shows part of the amino acid sequence of the P. chrysogenum HMW polypeptide.

Sequence listing No. 3 shows part of the amino acid sequence of the P. chrysogenum LMW polypeptide.

Sequence listing No. 4 shows a 29-mer oligonucleotide probe, based upon the N-terminal amino acid sequence of the HMW polypeptide.

Sequence listing No. 5 shows the redox sequence typical for thioredoxins.

In accordance with the subject invention, an oxido reductase enzyme system is provided which can be used to obtain or to enhance β-lactam antibiotic production. The subject enzyme system is a naturally occurring enzyme system, which comprises two components, a high molecular weight polypeptide component and a low molecular weight polypeptide component, in isolated form and an electron carrier such as NADPH in a reduced state. As measured by SDS-PAGE, the high molecular weight (HMW) component of the oxido reductase system from P. chrysogenum has an apparent molecular weight of about 36 kDa, and the low molecular weight (LMW) component has an apparent molecular weight of about 12 kDa. The enzyme system is capable of forming free thiols in compounds containing disulphide bonds, in the presence of an electron donor such as NADPH.

The enzyme system will comprise two polypeptide components which, when combined in the presence of a suitable electron carrier in reduced form, are capable of maintaining enzymes and intermediates in a reduced state in vitro. Activity was demonstrated by the reduction of 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) by following the increase of absorbance at 412 nm at 25°C. Alternatively other substrates were used and the rate of reduction was measured by following the decrease of absorbance of NADPH at 340 nm. In this case activity is expressed as μmoles of NADPH/min.mg protein.

The subject enzyme system was obtained from P. chrysogenum, or from other yeasts, fungi or bacteria comprising the genes encoding the HMW or LMW component of P. chrysogenum after transfer and expression of the genes of the invention into said microorganism.

The polypeptide components involved in reductase activity have been purified from cell-free extracts of P. chrysogenum. P. chrysogenum was grown on, for instance, a complex medium with for example starch as the carbon source, or on defined media. The optimum enzyme activity is dependent on the strain and the medium used, and the time of harvest of the mycelium.

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The polypeptide compounds of the subject enzyme system was isolated from cell-free extracts by combining a variety of techniques, including molecular sieving, ion exchange chromatography and affinity chromatography. The enzyme system was extracted from P. chrysogenum by an initial disruption of the cells, followed by homogenization in a low ionic strength (1 to 50 mM) buffer (for example a Tris buffer) at approximately pH 8.0. Lysis of cells can be performed in several ways, for example by use of a Braun dismembrator, by a French press or by mortaring of lyophilized mycelium followed by extraction in buffer (preferably of low ionic strength such as 1-50 mM Tris). After separating the liquid from the solid matter, nucleic acid was removed, for instance by compounds such as streptomycin sulphate, Polymin B or protamine sulphate. Protein in the liquid phase was precipitated using, for example, ammonium sulphate, pH 8.0. The precipitate forming between 50%-80% saturation was collected then dissolved and active fractions containing the enzyme system were collected. After fractionation of these active fractions by molecular sieving techniques (for example, low pressure resins, AcA54 or Sephadex G75, which fractionate approximately between 3 and 80 kDalton range) the activity disappeared. Only after combining fractions eluting early from these columns with fractions eluting later, fractions containing the HMW and LMW components were identified using the DTNB assay.

The HMW and LMW components of the enzyme system were individually purified. The fractions containing the HMW component were further fractionated using anion exchange resins such as diethylamino ethyl (DEAE) or mono Q at low, medium or high pressure, for example TSK-DEAE-650, equilibrated with a low ionic strength buffer (for example 50 mM Tris-HCl pH 8.0, 1 mM EDTA). The HMW component was eluted with a salt gradient, for example ranging from 0-0.5 M NaCl. Fractions showing activity in the DTNB assay eluted between 0.2 and 0.4 M NaCl with this gradient. After this step the HMW component is separated from the enzyme glutathione reductase which elutes at lower salt concentrations. The pooled fractions were further purified by affinity chromatography resins having affinity for NAD$^+$ or NADP$^+$, such as Blue Sepharose CL-6B. The resin was washed by a low ionic strength buffer of pH below 7.3, for example 20 mM Tris, 1 mM EDTA. The HMW component is eluted from the resin by adding 5 mM NADPH to the same buffer.

The fractions of the molecular sieving column which contain the LMW component were further purified using anion exchange resins such as DEAE or mono Q at low, medium or high pressure, for example TSK-DEAE-650S, equilibrated with a low ionic strength buffer, preferably 10 mM Tris-HCl pH 8.0, 1 mM EDTA or lower. The LMW component was eluted with a salt gradient, for example ranging from 0-0.3 M NaCl. To stabilize the activity of the LMW component, the fractions containing this component received a reducing agent, for instance DTT or β-mercaptoethanol. Before testing of the activity the reducing agent is always removed, for instance by extensive washing of the LMW component bound to a DEAE resin, by gel filtration, or by dialysis of the eluted fractions against a low ionic strength buffer such as 1-40 mM sodium acetate. The LMW component was further purified using cation exchange resins such as carboxymethyl (CM-)Sepharose equilibrated with a low ionic strength buffer with a pH below 5.5 (for example 20 mM sodium acetate pH 4.9 containing 1 mM EDTA). The LMW component was eluted with a gradient for example 0-600 mM sodium acetate. Fractions containing the LMW polypeptide eluted between 250 and 450 mM acetate.

The purity of the HMW and LMW component was examined by SDS-PAGE. Amino acid sequencing of these bands showed that these polypeptides were substantially free of cellular debris and other microorganism proteins, as judged by SDS-PAGE followed by amino acid sequencing.

After Blue Sepharose CL-6B affinity chromatography the HMW component has a specific activity in the range of 1.0-2.0 μmoles NADPH/min.mg prot. as determined with DTNB as a substrate and a fixed amount of DEAE purified LMW component. After CM-Sepharose chromatography the LMW component has a specific activity of 0.3-0.4 μmoles NADPH/min.mg prot. as determined with DTNB as a substrate. However, the measurement of specific activity is very dependent on the degree of purity of the HMW and LMW components, on the moment after purification at which the assay is conducted, and on the relative amounts of HMW and LMW components added to the reaction mixture. Highly purified polypeptide components are less stable than partially purified polypeptide components.

The enzyme system was able to reduce low molecular weight disulphides such as bis-ACV, DTNB, bis-(cys-gly) and bis-(Coenzyme A). Michaelis Menten constants (K_M) were measured for these substrates, using HMW and LMW components obtained after DEAE chromatography (18 and 2.5 μg, respectively). The K_M values were in the order of 125 μM for bis-ACV, 1.4 μM for DTNB, 83 μM for bis-(Coenzyme A) and 800 μM for bis-(cys-gly).

The enzyme system was also able to reduce protein disulphides such as in insulin. The K_M value for insulin, based on the molecular weight of the native molecule which contains three disulphides, is 2.3 mM.

Amino acid sequences of the HMW and LMW components isolated from P. chrysogenum were

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determined by methods known in the art. The HMW gene product comprises the amino acid sequence:

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Met-Val-His-Ser-Lys-Val-Val-Ile-Ile-Gly-
Ser-Gly-Pro-Gly-Ala-His-Thr-Ala-Ala-Ile-
Tyr-Leu-Ser-Arg-Ala-Glu-Leu-Gln-Pro-Val-
Leu-Tyr-Glu-Gly-Met-Leu-.
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10 Residues 2-29 have been determined by amino acid sequence analysis of the purified protein. Nucleotide sequence data of the cloned HMW gene confirmed tentative assignments in this sequence and extended the sequence with residue 1 and residues 30-36.

The LMW gene product comprises the amino acid sequence:

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(Gly)-Val-Thr-Pro-Ile-Lys-Ser-Val-Ala-Glu-
Tyr-Lys-Glu-Lys-Val-Thr-Asp-Ala-(Thr)-(Gly)-
Pro-Val-(Val)-(Val)-Asp-Phe-His-Ala-Thr-(Trp)-
(Glu)-Gly-Pro-(Xaa)-(Lys)-(Ala)-(Ile)-(Ala)-(Pro)-
(Glu)-.
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Some of the amino acids could not be assigned beyond a reasonable doubt; these tentative assignments are given in parentheses.

The invention also includes polypeptides with different amino acid sequences which are homologous to the HMW and LMW polypeptides of P. chrysogenum or parts thereof and show an comparable activity. 25 Homology is defined herein as a similarity score of at least 80% when compared to the amino acid sequence of the HMW or LMW protein, as determined by the BestFit programme of the Wisconsin Sequence Analysis Software package (version 6.0, release 1989, GCG, University of Wisconsin, U.S.A.) using parameter settings gap weight 3.000 and length weight 0.100. Amino acid sequences with similarity scores less than 85% but higher than 60% are termed similar herein. Homologous proteins may be isolated 30 from natural sources, or may be produced by mutagenesis of the HMW and LMW encoding genes.

Comparison of the amino acid sequence with known amino acid sequences revealed that the N-terminal amino acid sequence of the HMW polypeptide is similar to the N-terminal amino acid sequence of thioredoxin reductase of E. coli (EC 1.6.4.5) (84% similarity in an 33 amino acid overlap). The LMW sequence may contain the highly conserved redox sequence Cys-Gly-Pro-Cys typical for thioredoxins and 35 may be similar to thioredoxins obtained from several organisms (60% similarity in an 38 amino acid overlap to thioredoxin of E. coli. The finding that the LMW component could be replaced by thioredoxin obtained from Spirulina platensis, in an assay in which either DTNB or bis-ACV were reduced by the combined action of HMW and LMW component and NADPH, further indicates the homology of the LMW component with thioredoxin.

40 Thus it is highly probable that the high molecular weight polypeptide and the low molecular weight polypeptide isolated from P. chrysogenum can be referred to as the thioredoxin reductase and the thioredoxin of P. chrysogenum, respectively.

The polypeptides or parts thereof may be joined to other compounds, such as antigens, receptors, labels or the like.

45 Also provided for in this invention are the genes encoding the polypeptide components of the oxido reductase system of P. chrysogenum. The N-terminal amino acid sequences of the two polypeptides have been determined. This enables, using the approach of reverse genetics defined herein below, the isolation of genes which encode these polypeptides, and subsequently the determination of the nucleotide sequences of these genes (Miller and Ingolia, supra). In the reverse genetics approach, oligonucleotide probes 50 are designed based on a partial amino acid sequence from the N-terminus or internal peptide fragments of the purified enzyme (see e.g., Maniatis et al., Molecular Cloning (2nd ed.) 1989). These oligonucleotide probes may be used directly to screen genomic or cDNA libraries for the gene of interest. An example of such a library, containing DNA fragments of P. chrysogenum in the lambda phage vector EMBL-3 has been described in EP 0354624.

55 Alternatively, a set of synthetic oligonucleotide probes may be designed to be used in a polymerase chain reaction (PCR) using genomic DNA, cDNA or mRNA from the microorganism of interest to generate a larger probe fragment.

In another approach antibodies raised to the purified enzyme, or parts thereof, may be used to screen

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expression libraries for the gene of interest. Once genes, or part of the genes encoding the HMW and LMW polypeptides have been cloned, these genes can be used as probes to isolate the corresponding genes from other organisms by heterologous hybridization. These probes can be restriction fragments derived from DNA isolated from, for example, P. chrysogenum. Alternatively, synthetic oligonucleotide probes can

5 be made for instance comprising a region of the most conserved sequences in the gene. These oligonucleotide sequences can be used for screening of genomic or cDNA libraries. On the other hand, oligonucleotides may be made which can be used to isolate the gene of interest, or part of it, from genomic DNA, cDNA or mRNA of the microorganism of choice by using the PCR technique.

In yet another approach, cloned sequences of the genes encoding the HMW and LMW polypeptides

10 may be used to create oxido reductase mutants by gene disruption of the authentic HMW and LMW polypeptide encoding genes by transformation with cloned mutant derivatives of these genes. The resulting thioredoxin or thioredoxin reductase mutant can subsequently be used as a host for the selection and isolation of DNA fragments from the same or another organism which are able to restore oxido reductase activity.

15 Probes may be modified by conjugation to a variety of labels which allow for detection of duplex formation between the probe and its complementary target. Labels include radioactive isotopes, ligands, e.g., biotin, enzymes, fluorescers and the like. A wide variety of protocols for labelling probes and detecting duplexes formed between probes and their target hybridization sequences have been described in the literature. See for example, Berger and Kimmel, editors, Guide to Molecular Cloning Techniques (1987),

20 Academic Press Inc., San Diego, CA. Probe sequences may also be joined to a variety of other nucleic acid sequences. Among these other nucleic acid sequences are vectors such as plasmids, cosmids, phages, and the like. By joining the probe sequence to a vector sequence, probes may be conveniently created, expanded, stored, and modified.

Convenient restriction sites may be designed into the DNA sequences encoding the enzyme system;

25 when possible, the restriction site(s) leave(s) the amino acid sequence of the expression product unaltered. In some cases, incorporation of new restriction sites may yield an altered amino acid sequence. However, it is highly desirable that the three-dimensional structure of the HMW and LMW polypeptides be retained, particularly that (those) portion(s) of the structure of the enzyme system which may be responsible for enzyme activity.

30 The invention also includes genes comprising different nucleotide sequences for instance with conservative mutations, where the sequence encodes the same amino acid sequence, but may have as many as 30% different bases, more usually not more than 10% different bases, or mutations which are non-conservative, where fewer than about 10%, more usually fewer than about 5%, and preferably not more than 1% of the amino acids are substituted or deleted, and there are fewer than 5% of inserted amino

35 acids, where the percent is based on the number of naturally occurring amino acids. Said genes may also be isolated by hybridization.

Once having isolated a DNA sequence encoding the enzyme system, the DNA sequence may then be used for expression in a convenient host, either prokaryotic or eukaryotic. For expression of the sequence, where necessary an initial methionine codon is provided. Where the DNA sequence encoding the enzyme

40 system is to be expressed in a host which recognizes efficiently the authentic transcriptional and translational regulatory regions of the native enzyme system encoding gene(s), the entire gene(s) with its authentic 5' and 3'-regulatory regions may be introduced into the host without further manipulation.

Where the DNA sequence encoding the enzyme system is to be expressed in a host cell which does not recognize the authentic expression signals or where the native gene encoding the enzyme system is not

45 being used, further manipulation may be required. The non-coding 5'-region upstream from the open reading frame (ORF) may be removed by exonuclease digestion, for instance by Bal31 digestion, or the like. Alternatively, where a convenient restriction site is present near the 5'- terminus of the ORF, the DNA may be restricted and an adapter employed for linking the ORF to a DNA sequence providing expression of the ORF in the host cell, where the adapter provides for the lost nucleotides of the ORF. Similarly the

50 authentic 3'-transcriptional regulatory regions can be replaced by 3'-transcriptional regulatory regions functional in the host. In addition, the expression in other hosts, especially prokaryotic ones, may require the removal of introns in the gene, or the use of cDNA copies of the gene.

For the genes encoding the component polypeptides of the enzyme system, the original expression signals can be replaced by expression signals obtained from the same or another organism, which are

55 functional in the host cell, for example, in E. coli or in Streptomyces, or in filamentous fungi such as P. chrysogenum. These promoters preferably are expressed during β-lactam synthesis. Expression signals are defined herein as signals necessary and sufficient for efficient initiation and termination of transcription and efficient initiation and termination of translation.

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The genes may be introduced into the host cell in accordance with known techniques, such as transformation, transfection by contacting the cells with a virus, micro-injection of the genes into the cells and the like. The genes can be incorporated in constructs, either together in one construct, or each in a separate construct. The genes can be introduced into strains of P. chrysogenum or other β-lactam producing organisms, preferably production strains, by (co)transformation with a suitable selection marker. It is obvious to those skilled in the art that selection of transformants can be achieved by using different selection markers, homologous or heterologous to the host, in the presence or absence of vector sequences, physically linked or not to the non-selectable DNA. Particularly, the microbial hosts are selected from the group of fungi, preferably Penicillium chrysogenum, Acremonium chrysogenum or Aspergillus nidulans, or from the group of Streptomyces.

In use for β-lactam production the isolated enzyme system of P. chrysogenum finds several applications. Next to the polypeptide components of the P. chrysogenum system, also thioredoxin and thioredoxin reductase obtained from other organisms can be used. This is exemplified by the finding that the LMW component can be replaced by thioredoxin obtained from Spirulina platensis, in an assay in which either DTNB or bis-ACV are reduced by the combined action of HMW and LMW component and NADPH. Thioredoxin is defined as a protein with two redox-active half cystine residues in an exposed active centre, having the amino acid sequence: -Cys-Gly-Pro-Cys-, and which is able to reduce, besides other compounds containing disulphide bonds, disulphide bonds in proteins (A. Holmgren, Ann. Rev. Biochem. (1985) 54:237-271). The polypeptide components do not have to be purified completely, for most applications partially purified polypeptide components can be used.

The enzyme system can replace DTT. DTT is used to provide reducing conditions, for instance in processes where isolated enzymes are used to produce β-lactam compounds. The activity of most of the β-lactam biosynthetic enzymes is stimulated by the presence of DTT. The tripeptide ACV, the substrate of IPNS, has to be in a reduced state as well. However, in the presence of iron DTT will inactivate many enzymes (Kim et al., J. Biol. Chem. (1985) 260:15394-15397). Iron is an essential cofactor for the enzymes involved in β-lactam synthesis, such as catalyzing ringclosure, ringexpansion and hydroxylation. It has been exemplified that the thioredoxin system can replace DTT to provide reducing conditions for β-lactam biosynthesis. In the presence of the oxido reductase system obtainable from P. chrysogenum and the thioredoxin systems already known, isopenicillin N was produced from ACV disulphide under non-reducing conditions.

The LMW component of all those enzyme systems can be used to enhance the activity of β-lactam biosynthetic enzymes which are dependent on the stimulating effect of DTT. This has been exemplified for ACVS. Addition of thioredoxin and DTT to the enzyme resulted in higher activity than the addition of DTT alone. Thioredoxin reductase is defined as a protein which is able to reduce thioredoxin in the presence of a suitable electron donor.

Thus a process for reducing disulphide compounds and compounds involved in the production of β-lactam derivatives in vitro has been provided for by contacting, in the presence of an electron donor, the compounds to be reduced with a composition comprising the oxido reductase system or components, wherein said enzyme system is obtainable from P. chrysogenum, and has as components a high molecular weight (HMW) polypeptide with a molecular weight of about 36 kDa and a low molecular weight (LMW) polypeptide with a molecular weight of about 12 kDa, and being capable of reducing disulphide compounds, or with a LMW polypeptide in the presence of a reducing agent as for instance DTT, or wherein said enzyme system or components are a thioredoxin system, or thioredoxin and a thioredoxin reductase, obtainable from any organism.

In use, the genes encoding the polypeptide components of the oxido reductase system of P. chrysogenum find several applications. Rather than isolate the enzyme system from natural sources, the HMW and/or LMW components may be prepared from microorganisms modified by recombinant techniques. This may be accomplished by preparing a DNA construct comprising one or more genes encoding the HMW and/or the LMW polypeptide component of the oxido reductase system wherein optionally one or both of the expression signals has been replaced by expression signals obtained from the same or another organism, as described above; and transforming a host organism with these DNA constructs. The enzyme system or components thereof may be isolated from transformed hosts essentially according to methods provided above.

By use of cloned HMW and LMW genes or cloned genes encoding thioredoxin and thioredoxin reductase, modified enzymes may be designed and synthesized. The DNA of the invention may also be modified by known techniques of site-directed mutagenesis to yield DNA in which specific mutations, deletions and insertions have been introduced. These modifications will result in modified characteristics of the enzymes, such as a change in pH or temperature optimum, a change in stability or a change in

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substrate specificity. The latter could for instance result in an enzyme with a higher affinity for other components of the oxido reductase system, for β-lactam biosynthetic enzymes or for disulphide compounds, for instance ACV, involved in the production of β-lactam compounds, or a combination thereof. The host strains, transformed with genes encoding these modified enzymes may be able to produce β-lactam antibiotics at higher levels as compared to non-transformed strains.

The genes encoding the polypeptide components of all thioredoxin systems including the oxido reductase system of P. chrysogenum may be used for enhancing the production of β-lactam compounds in a microbial host. DNA constructs prepared as described in this invention are used to transform microorganisms producing said compounds. Preferred microorganisms for transformation are eukaryotic β-lactam producers such as P. chrysogenum, A. chrysogenum or Aspergillus species, and prokaryotic β-lactam producers such as Flavobacterium or Streptomyces species.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit and scope of the appended claims.

The following non-limitative examples will further illustrate the invention.

All documents cited are incorporated herein by reference.

EXPERIMENTAL

Example 1

Demonstration of DTNB reducing activity by combining high and a low molecular weight fraction of extracts of Penicillium chrysogenum

Culturing:

Rice containing spores of P. chrysogenum strain NRRL 1951 was prepared as described by Lein et al. (Lein (1986) in Vanek and Hostalek (ed.) Buttersworth, Boston, pp. 105-139, Overproduction of microbial metabolites: strain improvement and process control strategies). Tryptic soy broth (TSB) with 1% starch as the carbon source was inoculated with 10^5 conidia per ml. After growing for 24-48 h at 30°C 5 ml of this culture was used to inoculate 500 ml of TSB-starch medium. After 24 h, the mycelia were harvested by filtration and the resulting filter cake was washed twice with 0.9% NaCl. Packed mycelia were kept frozen at -20°C until use.

DTNB reductase activity assay:

DTNB reductase activity was measured as follows: The reaction mixture contained (final concentration) 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.02 mM DTNB, 0.2 mM NADPH. Enzyme preparations were added to a final volume of 1 ml. The reaction was followed by measuring the increase of absorbance at 412 nm at 25°C during the first three minutes or longer if necessary. Control experiments were included in which the enzyme preparations or NADPH were omitted.

Preparation of cell fractions:

10 g (wet weight) of mycelia were suspended in 0.05 M Tris-HCl buffer pH 8.0, 1 mM EDTA (TE-buffer) to a total volume of 50 ml and disrupted in a Braun dismembrator (Braun, Melsungen, FRG) using Ballotini glass beads (Sigma type V, diameter 450-500 μm) for periods of 30 s at intervals of 15 s with refrigeration. The homogenized suspension then was cleared by passing over a glass wool filter. This step and the following ones were carried out at 4°C. To the cell-free extract, 10% (w/v) streptomycin sulphate in TE-buffer was added slowly to a final concentration of 1%. After stirring for 45 min in the cold, the nucleic acid precipitate was removed by centrifugation at 10,000 x g for 10 min. The supernatant was fractionated by precipitation with ammonium sulphate while maintaining the pH of the solution at 8.0 during ammonium sulphate additions. The fraction precipitating between 50-80% saturation was dissolved in a small volume of TE-buffer. The dissolved fraction was applied to an AcA54 gel-filtration column (2.6 x 35 cm) equilibrated with TE-buffer. The column was eluted with TE-buffer at a flow rate of 0.5 ml/min and fractions of 3 ml were collected.

DTNB reductase activity was present in the ammonium sulphate pellet precipitating between 50-80%

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saturation, but was absent in the separate fraction from the AcA54 gel-filtration column. DTNB reductase activity was restored by adding particular fractions containing LMW material (<15 kDa) to particular fractions containing HMW material (>15 kDa). The DTNB reductase activity was found to be dependent on the presence of NADPH. It can be concluded from these experiments that DTNB reduction by cell-free extracts of P. chrysogenum is achieved by at least two polypeptide components (HMW and LMW components) and an electron donor.

Example 2

Purification of the HMW and LMW components of the oxido reductase system involved in the reduction of DTNB

DTNB reductase activity assay:

The assay conditions for the purification of the HMW component were as described in Example 1. The reaction mixture was prepared as follows. For the purification of the HMW component, 50-100 μl AcA54 gel-filtration fractions containing LMW material (<15 kDa) were included. For the purification of the LMW component, 25-50 μl of gel-filtration fractions containing HMW material (>15 kDa) were included in the mixture.

Purification of the HMW component:

Gel-filtration fractions which contained HMW material and which were active in the DTNB assay were pooled and applied to a TSK-DEAE-650 (Merck) column (10 ml bed volume) which was previously equilibrated with TE-buffer. The column was eluted with a linear gradient of 0 to 0.5 M NaCl. Fractions eluting at 0.25 to 0.32 M NaCl were active in the DTNB reductase assay. These fractions were subsequently loaded on a Blue Sepharose CL-6B column. Fractions containing HMW were eluted with 5 mM NADPH in 20 mM Tris-HCl buffer pH 7.25 containing 1 mM EDTA. This procedure resulted in a fraction containing one polypeptide band with an apparent molecular mass of 36 kDa as determined by SDS-PAGE (Laemmli, Nature (1970) 227:680-685).

Purification of the LMW component:

Gel-filtration fractions which contained LMW material and which were active in the DTNB reductase assay were pooled, incubated with 2 mM DTT and applied to a TSK-DEAE-650S) (Merck) column with 10 ml bed volume, which was previously equilibrated with 10 mM TE-buffer. The column was eluted with a linear gradient 0-0.3 M NaCl. Fractions eluting at 0.1 to 0.15 M NaCl were active in the DTNB reductase assay. These fractions were incubated with 2 mM DTT for 30 min. After dialysis against 20 mM sodium acetate buffer pH 4.9 containing 1 mM EDTA, the combined fractions were loaded on CM-Sepharose, 10 ml bed volume, equilibrated in the same buffer. The column was eluted with a linear gradient of 40-600 mM sodium acetate in the same buffer. Fractions eluting between 300-400 mM acetate buffer were active in DTNB reductase assay. This procedure resulted in a fraction containing a polypeptide band with an apparent molecular mass of 12 kDa as determined by SDS-PAGE.

Example 3

Determination of amino acid sequences of the purified HMW and LMW components

The purified LMW component was applied to a 17.5% SDS-PAA gel, the purified HMW component to a 12% SDS-PAA gel. After running of the gels, the polypeptides were electrophoretically transferred to a polyvinylidene difluoride membrane. The membrane was stained with 0.1% Coomassie Brilliant Blue R250 in 50% methanol, 10% acetic acid in water and subsequently destained. The polypeptide bands corresponding to the HMW and the LMW component were analyzed for N-terminal amino acid sequencing, using a gas phase sequenator (Applied Biosystems model 470 a).

The following amino acid sequence was determined for the HMW polypeptide:

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```
(Val)-(His)-Ser-Lys-Val-Val-Ile-Ile-Gly-Ser-
Gly-Pro-Gly-Ala-His-Thr-Ala-Ala-Ile-Tyr-
Leu-Ser-(Arg)-Ala-Glu-Leu-Gln-Pro-.
```

Tentative assignments are given in parenthesis.
The following amino acid sequence was determined for the LMW polypeptide:

```
(Gly)-Val-Thr-Pro-Ile-Lys-Ser-Val-Ala-Glu-
Tyr-Lys-Glu-Lys-Val-Thr-Asp-Ala-(Thr)-(Gly)-
Pro-Val-(Val)-(Val)-Asp-Phe-His-Ala-Thr-(Trp)-
(Glu)-Gly-Pro-(Xaa)-(Lys)-(Ala)-(Ile)-(Ala)-(Pro)-
(Glu)-.
```

Tentative assignments are given in parentheses.

Comparison of these amino acid sequences with known amino acid sequences and nucleotide sequences can be performed by a library search on the protein data bases of the National Biomedical Research Foundation (updated to March 1990) and the Swiss Protein Library (updated to April 1990), and the nucleotide sequence data bases of the European Molecular Biology Laboratory (updated to May 1990), using, respectively, the FastP and TFastA programmes described by Lipman and Pearson (Science (1985) 227: 1435-1441) and Pearson and Lipman (Proc. Natl. Acad. Sci. (USA) (1988) 85: 2444-2448). A more detailed comparison of the similarity between two sequences can be made by the Bestfit programme which uses an algoritm described by Smith and Waterman (Advances in Applied Mathematics, 1981, 2:482-489). The FastP, TFastA and BestFit programmes used are part of the GCG Sequence Analysis Software Package (version 6.0, release 1989, GCG, University of Wisconsin, U.S.A.). The search and comparison revealed that the HMW sequence is similar to the N-terminal amino acid sequence of thioredoxin reductase of E. coli (EC 1.6.4.5) (84% similarity in a sequence of 27 amino acids overlap).

No significant similarity is found for the LMW sequence 2-16 in the above-indicated libraries. Therefore, the oxido reductase system having the above sequences and characteristics is a novel system.

The extended LMW sequence 16-38 is tentative at several positions. Nevertheless, it was recognized that residues 31-34 form a putative consensus sequence Cys-Gly-Pro-Cys, typically involved in redox reactions. A library search and comparison performed with LMW sequence 2-38 confirmed this notion by revealing similarity of this sequence to thioredoxin of different species (for instance 60% similarity in a 38 amino acid overlap with thioredoxin of E. coli).

Example 4

Demonstration of the reduction of bis-ACV by the combined action of the HMW and LMW component

Bis-ACV reductase assay:

The reaction mixture contained 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mg bis-ACV (Bachem Feinchemikalien AG, Bubendorf, Switzerland), 0.1 mM NADPH and purified HMW and LMW components. The assay volume was 1 ml and incubation was at 30° C. The reaction was followed at 340 nm.

Control experiments were included in which either the HMW or the LMW component, or bis-ACV was omitted. Whereas no activity was found in any of the control experiments, a decrease of absorbance at 340 nm, due to a consumption of NADPH, was observed in reaction mixtures which contained all of the above components.

Example 5

Demonstration of the reduction of bis-ACV and DTNB by the combined action of the HMW component obtained from P. chrysogenum and thioredoxin obtained from Spirulina platensis

The DTNB reductase assay was done as described in Example 2, except that both HMW and LMW components used were purified until after DEAE chromatography. The amount of protein present in the reaction mixuture was 0.264 mg of the HMW component and either 20 μg of the LMW component or 10 μg

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of the thioredoxin obtained from Spirulina platensis (Sigma, St. Louis, U.S.A.). In this experiment the increase of absorbance was measured at 412 nm. After 15 minutes of incubation the increase of absorbance was 0.225 for the combination of HMW and LMW, and 0.080 for the combination of HMW and thioredoxin. Control experiments in which either the HMW or the LMW component were omitted, or in which either the HMW or the thioredoxin were omitted, did not show an increase of absorbance.

Reduction of bis-ACV by the combined action of the HWM compound of P. chrysogenum and thioredoxin of Spirulina platensis was determined as follows. The reaction mixture contained 80 mM Tris-HCl pH 8.0, 1.5 mM EDTA, 0.03 mg/ml bis-ACV and 0.1 mM NADPH. The amounts of the proteins added are given in Table 1. The HMW and LMW components were purified until after DEAE chromatography. The assay volume was 0.5 ml and incubation was at room temperature. The reaction was followed at 340 nm and activity was expressed as μmoles of NADPH oxidized per minute.

Table 1

HMW (μg)	LMW (μg)	thioredoxin (μg)	μmol NADPH/minute
250	10	0	2.25
250	5	0	1.13
60	0	4	1.93
250	0	2	0.64
250	0	1	0.32
125	0	0	0.32
0	10	0	0.16
0	0	4	0.16

The results show that thioredoxin of Spirulina platensis has affinity for the substrate bis-ACV. The activity was clearly dependent on the concentration of thioredoxin used. Moreover, the results show that the HMW component of P. chrysogenum has affinity for thioredoxin obtained from Spirulina platensis.

Example 6

Demonstration of the production of isopenicillin N from bis-ACV under non-reducing conditions

The enzymatic synthesis of isopenicilin N is dependent on the presence of the thiol form of ACV. In this example the synthesis of isopenicillin N by IPNS was performed with bis-ACV as a substrate. The assay conditions for the production of isopenicillin N were as follows. The reaction mixture contained 50 mM Tris-HCl pH 7.5, 1.42 mM bis-ACV, 3.84 mM Ascorbate, 0.8 mM Fe^{2+}, 0.7 mM EDTA, 14.28 mM NADPH, 16.8 μg of IPNS and HMW and LMW components as required. Enzyme preparations were added to a final volume of 70 μl. The IPNS was a preparation made from an E. coli strain expressing the IPNS gene from Flavobacterium sp.12.154. The IPNS gene has been cloned into the PIN OMPA expression vector (F.M. Ausubel et al. (eds) Current Protocols in Molecular Biology (1987-1988), John Wiley and Son, New York). E. coli MC1022 has been transformed with the resulting vector pDS510.

E. coli MC1022 containing pDS510 was grown in LB medium + 50 μg/ml kanamycin over night. The cells were diluted 100 fold into LB + 50 μg/ml kanamycin + 0.1 mM isopropylthiogalactoside (IPTG), and grown in 2 liter flasks. The cells were harvested after 6 hours; the OD_{600} was approximately 2. The cells were collected by centrifugation and washed in 50 mM Tris-HCl, pH 7.5. The cells were sonicated in the same tris buffer containing 1 mM DTT. The crude extract was treated with 1% streptomycin sulphate to precipitate nucleic acids. An ammonium sulphate fraction precipitating between 40-80% saturation was collected. The sample was run on an AcA44 column (55 cm x 2.6 cm), at 0.33 ml/min, 12 min. Antibiotic activity in the fractions was assayed in the standard bioassay employing Micrococcus luteus as the test organism. A standard curve of different cephalosporin C concentrations was formed, thus the numbers express activity relative to that of cephalosporin C. Active fractions were pooled and loaded on a DEAE-Sepharose column 1.6 cm x 10 cm. Proteins were eluted with a 150 ml gradient of 0 to 400 mM NaCl. The flow was 0.33 ml/min. Fractions were collected every 7.5 min. The fractions were assayed and fractions showing high activity were pooled. This IPNS preparation was used for further experiments.

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The HMW and LMW components were partially purified including DEAE chromatography as described in Example 2.

The formation of isopenicillin N from bis-ACV in the presence of the oxido reductase system from P. chrysogenum was determined. Antibiotic activity was determined as described above. Control experiments were included in which either the HMW or the LMW component or NADPH were omitted. The results of a typical experiment were as follows.

Production of isopenicillin N from bis-ACV under non-reducing conditions

HMW	LMW	NADPH	Zone of inhib.	Antibiotic
μg/reaction			mm	μg/ml
6.6	8	−	ND	ND
39.6	8	−	ND	ND
145.2	11	−	ND	ND
−	8	+	ND	ND
−	11	+	ND	ND
6.6	−	+	ND	ND
39.8	−	+	ND	ND
105.6	−	+	ND	ND
145.2	−	+	ND	ND
6.6	8	+	7	38
19.8	8	+	12	72
39.6	8	+	12	72
105.6	8	+	ND	ND
145.2	11	+	ND	ND

ND = not detected

The production of antibiotic was not detected in any of the control experiments. When both the HMW and the LMW components and NADPH were present, antibiotic activity was detected. The synthesis of isopenicillin N appeared to be inhibited by high concentrations of the HMW component which may be due to impurities in the preparation.

This example demonstrates that the oxido reductase system of P. chrysogenum provides the reducing environment necessary for the production of isopenicillin N from bis-ACV by IPNS.

Example 7

Demonstration of enhanced activity of ACVS by contacting with the LMW component

Purification of ACVS:

ACVS was purified from Acremonium chrysogenum C10 (ATCC 48272) as essentially described by Baldwin et al. (J. Antibiotics (1991) 44:241-248).

The ammonium sulphate precipitate was dissolved and applied onto a gel-filtration column (Ultrogel AcA34). The ACVS activity assay was done as described by Van Liempt et al. (J. Biol. Chem. (1989) 264:3680-3684). Fractions showing high activity of ACVS were collected (75-85 μg protein/ml). The preparation shows a purity of 80% for ACVS as judged after SDS-PAGE and staining with Coomassie Brilliant Blue R250. DTE was removed by passing the enzyme through a PD10 column just before use in further experiments.

Purification of the LMW component:

The LMW component was obtained from P. chrysogenum strain Wisconsin 54-1255 (ATCC 28089). The LMW component was purified as described in Example 2. The fractions obtained after DEAE chromatography which were active in the DTNB assay, were used for further experiments.

Determination of ACVS activity in the presence of DTT and LMW component:

The ACVS activity of a reaction mixture containing ACVS and fresh DTT was compared to the activity of a reaction mixture containing ACVS, fresh DTT and the LMW component. As a control for ACVS activity ATP was omitted from the reaction mixture. To compensate for lower protein concentrations in the reaction mixture bovin serum albumin (BSA) was added.

The reaction mixture contained 150 μl of the ACVS preparation. DTT was added to a final concentration of 1.7 mM. ATP was added optionally to a final concentration of 5 mM. Either 25 μl of 0.5 mg/ml BSA solution or 12.5 μl of the same solution together with 12.5 μl of a 1.5 mg/ml LMW component preparation was added. The reaction mixture was allowed to stand for 10 min at room temperature. The reaction was started by the addition of 50 μl of a mixture containing L-α-aminoadipic acid (final concentration 1.3 mM), L-cysteine (1.3 mM), MgCl$_2$ (25 mM), Tris-HCl (350 mM) pH 7.5, EDTA (0.1 mM), glycerol (9% w/w) and 0.25 μC L-[U-^{14}C]valine (3 μM). The reaction was allowed to proceed for 30 min at 30° C. The reaction was stopped by the addition of 250 μl of a stop mixture (80 μM ACV, 0.3 mM DTT, 0.5 mM L-valine and 10% trichloroacetic acid (TCA)). After separation of the labelled valine by a passage through Pora pak Q (Waters Associated Inc. Milford, MA), the dpm-values given below correspond to the ACV produced during incubation.

The results are given in Table 2.

Table 2

ATP	DTT	LMW	dpm
−	−	−	180
+	−	−	198
+	+	−	278
+	+	+	509

Similar experiments with LMW component derived from a gel filtration through Ultrogel AcA54 containing no salt showed an even stronger effect on ACVS activity (2.3 fold stimulation). Thus the LMW component stimulates ACVS activity.

Example 8

Isolation of the gene encoding the HMW polypeptide

Based upon the N-terminal amino acid sequence of the HMW polypeptide a 29-mer oligonucleotide probe has been synthesized with the following mixed nucleotide sequence:

```
5'-GGA CCI GGA GCI CAC ACI GCI GCI ATC GC-3'
     C       C       G               T
     T       T
```
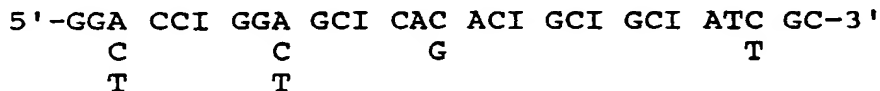
where A is deoxyadenyl, G is deoxyguanyl, C is deoxycytidyl, T is deoxythymidyl and I is deoxyinosityl.

The rationale for designing deoxyinosityl containing probes is twofold: to expand the number of sequences in the probe to cover all possible codon usages, and to restrict the number of different sequences in the mixture to a reasonable one. Deoxyinosityl itself is considered to be essentially neutral in its effects on forming DNA hybrids.

The techniques and procedures employed in the following process of isolation of the HMW gene are well known in the art and have been adequately described by Maniatis et al. (supra).

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The HMW oligonucleotide probe was labelled with gamma-[^{22}P]-ATP and T4 polynucleotide kinase. The labelled probe was used to screen a genomic library of P. chrysogenum, made in lambda phage vector EMBL-3. Plague hybridization was performed on approximately 10^4 plagues on nitrocellulose filters. Hybridization was performed at 42°C in a standard hybridization solution containing 15% formamide. Filters

5 were washed with 1-2 x SSC (1 x SSC is: 0.15 M sodium chloride/15 mM sodium citrate) at 50-60°C. In these experiments eight positively responding plaques were found. Five of these were chosen for a second round of screening with the same HMW probe. All five phage isolates gave a clear positive response. Well separated strongly responding plaques were picked for further characterization. Small scale DNA preparations of the recombinant phages were made for each isolate.

10 Purified DNAs were digested with restriction enzymes and, following agarose gel electrophoresis, Southern blots were prepared according to procedures well known in the art (Maniatis et al., 1989, supra). Hybridization of Southern blots with the HMW probe revealed, for all five lambda phages, positive hybridization of the probe to a unique restriction fragment. SalI restriction fragments sized 4.7 kilobases (kb), 8.0 kb and 6.2 kb derived from lambda phages 1, 4 and 5, respectively, were isolated and subcloned

15 into E. coli vector pUC19 (Pouwels et al., Cloning Vectors, a laboratory manual, Elsevier, Amsterdam, 1987). The resulting plasmids, termed pPCH-1, pPCH-4 and pPCH-5, respectively, have been further analyzed by restriction enzyme analysis and Southern blot hybridization. All three plasmids contained a 1.2 kb EcoRV restriction fragment responding to the HMW probe. A partial restriction map of the 4.7 kb SalI fragment contained in pPCH-1 is shown in Figure 1. A 0.5 kb HindIII-BamHI restriction fragment was identified in

20 pPCH-1, responding to the HMW probe. This fragment was isolated from pPHC-1 and subcloned into M13 sequence vector M13 mp 18/19 (Pouwels et al., supra). Determination of the nucleotide sequence of the HindIII-BamHI restriction fragment was subsequently performed following the procedure of Sanger (Maniatis et al., 1989, supra). The nucleotide sequence revealed an open reading frame encoding the N-terminal amino acid sequence of the HMW gene (Figure 2). The position of the 5'-end of the HMW gene, as well as

25 the direction of the transcription of the gene is indicated (Figure 1).

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

30 The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

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SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 250 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: DNA (genomic)

 (iii) HYPOTHETICAL: No

 (iv) ANTI-SENSE: No

 (vi) ORIGINAL SOURCE: Penicillium chrysogenum

 (ix) FEATURE:
  (A) NAME/KEY: reading frame of the HMW gene of the P. chrysogenum oxido
   reductase system
  (B) LOCATION: 59..80 and 164..250

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GCCTCGGTCT TCTCCAAGTC ATTTCCGAGC GTCGCGCGTC AATTCCAATA CCGCCAGA        58

ATG GTG CAC TCT AAA GTA GTC A|GT AAGTGGTTTT TCGAGGAGAT CGAATGATCC      112
Met Val His Ser Lys Val Val I|
 1           5

GGATCACCTA GATTTCTTCG ATATCAGACT TCTAACCGTT TTCCCCCACA G|TT ATT        168
                                                        |le Ile

GGC TCC GGC CCT GGT GCC CAC ACT GCC GCT ATC TAT CTA TCG CGC GCA       216
Gly Ser Gly Pro Gly Ala His Thr Ala Ala Ile Tyr Leu Ser Arg Ala
 10          15          20          25

GAG CTC CAG CCA GTC CTT TAC GAG GGC ATG CTC G                         250
Glu Leu Gln Pro Val Leu Tyr Glu Gly Met Leu
             30          35
```

(2) INFORMATION FOR SEQ ID NO:2:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 36 amino acids
  (B) TYPE: amino acid
  (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: protein

 (vi) ORIGINAL SOURCE: P. chrysogenum

 (ix) FEATURE:
  (A) NAME/KEY: HMW component of the P. chrysogenum oxido reductase system
  (B) LOCATION: 1..36

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Val His Ser Lys Val Val Ile Ile Gly Ser Gly Pro Gly Ala His
 1               5               10              15

Thr Ala Ala Ile Tyr Leu Ser Arg Ala Glu Leu Gln Pro Val Leu Tyr
                20              25              30

Glu Gly Met Leu
        35
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 40 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (vi) ORIGINAL SOURCE: P. chrysogenum

    (ix) FEATURE:
        (A) NAME/KEY: LMW component of the P. chrysogenum oxido reductase system
        (B) LOCATION: 1..40
        (C) OTHER INFORMATION: /label= alternativeaas
           /note= "aa1=probably Gly, aa19=probably Thr, aa20=probably Gly, aa23=probalby Val,
           aa24=probably Val, aa30=probably Trp, aa31=probably Glu, aa34=unknown, aa35=probably
           cys, aa36=probably Ala, aa37=probably Ile, aa38=probably Ala, aa39=probably Pro,
           aa40=probably Glu"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Xaa Val Thr Pro Ile Lys Ser Val Ala Glu Tyr Lys Glu Lys Val Thr
 1               5               10              15

Asp Ala Xaa Xaa Pro Val Xaa Xaa Asp Phe His Ala Thr Xaa Xaa Gly
                20              25              30

Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 29 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (synthetic)

    (ix) FEATURE:
        (C) OTHER INFORMATION: /label= alternativeaas
           /note= "base pair 3= A or C or T; base pair 9= A or C or T; base pair 15= C or G;
           base pair 21= C or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGHCCIGGHG CICASACIGC IGCIATYGC   29

(2) INFORMATION FOR SEQ ID NO:5:

       (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 4 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: protein

       (ix) FEATURE:
          (A) NAME/KEY: redox sequence typical for thioredoxins
          (B) LOCATION: 1..4

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Cys Gly Pro Cys
1

Claims

1. An oxido reductase system obtainable from P. chrysogenum, comprising in isolated form as first and second components two polypeptides, a high molecular weight (HMW) polypeptide with a molecular weight of about 36 kDa and a low molecular weight (LMW) polypeptide with a molecular weight of about 12 kDa and capable of reducing disulphide compounds in the presence of a reduced electron carrier.

2. An oxido reductase system according to claim 1, capable of reducing ACV in oxidized form, for instance bis-ACV.

3. An oxido reductase system according to Claim 1, wherein the N-terminal amino acid sequence for said first component is equal to or homologous with:

          Met-Val-His-Ser-Lys-Val-Val-Ile-Ile-Gly-
          Ser-Gly-Pro-Gly-Ala-His-Thr-Ala-Ala-Ile-
          Tyr-Leu-Ser-Arg-Ala-Glu-Leu-Gln-Pro-Val-
          Leu-Tyr-Glu-Gly-Met-Leu-

and the N-terminal amino acid sequence for said second component is equal to or homologous with:

          (Gly)-Val-Thr-Pro-Ile-Lys-Ser-Val-Ala-Glu-
          Tyr-Lys-Glu-Lys-Val-Thr-Asp-Ala-(Thr)-(Gly)-
          Pro-Val-(Val)-(Val)-Asp-Phe-His-Ala-Thr-(Trp)-
          (Glu)-Gly-Pro-(Xaa)-(Lys)-(Ala)-(Ile)-(Ala)-(Pro)-
          (Glu)-.

4. A process for producing an enzyme system according to Claim 1-3, comprising:
   growing a microorganism culture containing cells comprising DNA sequences encoding said first

and second components each sequence under the regulatory control of expression signals functional in said cells, whereby said components of said enzyme system are expressed; and isolating said enzyme system.

5. The oxido reductase system according to any one of the Claims 1-3, substantially free of cellular debris and other microorganism proteins.

6. A process for isolating an oxido reductase system according to any one of the Claims 1-3 and 5, said process comprising:

obtaining a cell-free extract of a microorganism comprising said system;

separating said cell-free extract into fractions by chromatography steps and isolating an active fraction after each of said chromatography steps either said first component or said second component, said steps including at least one of (a) molecular sieving chromatography; (b) anion exchange chromatography; (c) cation exchange chromatography; (d) affinity chromatography; wherein said steps may be performed in any sequence and are performed on an active fraction identified from a previous step; and following said (a)-(d) steps, optionally separating an active fraction from said (a)-(d) steps on SDS-PAGE.

7. A DNA sequence encoding at least a functional portion of a polypeptide component of an oxido reductase system obtainable from P. chrysogenum and capable of reducing disulphide compounds in the presence of a reduced electron donor.

8. The DNA sequence according to Claim 7, wherein the encoded HMW polypeptide component comprises the amino acid sequence:

```
Met-Val-His-Ser-Lys-Val-Val-Ile-Ile-Gly-
Ser-Gly-Pro-Gly-Ala-His-Thr-Ala-Ala-Ile-
Tyr-Leu-Ser-Arg-Ala-Glu-Leu-Gln-Pro-Val-
Leu-Tyr-Glu-Gly-Met-Leu-.
```

9. The DNA sequence according to Claim 7, wherein the encoded LMW polypeptide component comprises the amino acid sequence:

```
(Gly)-Val-Thr-Pro-Ile-Lys-Ser-Val-Ala-Glu-
Tyr-Lys-Glu-Lys-Val-Thr-Asp-Ala-(Thr)-(Gly)-
Pro-Val-(Val)-(Val)-Asp-Phe-His-Ala-Thr-(Trp)-
(Glu)-Gly-Pro-(Xaa)-(Lys)-(Ala)-(Ile)-(Ala)-(Pro)-
(Glu)-.
```

10. The DNA sequence according to any one of Claims 7-9, isolated from Penicillium chrysogenum.

11. The expression signals of the DNA sequence indicated in claim 10.

12. The DNA sequence according to any one of Claims 7-10, under the control of expression signals obtained from the same or another organism.

13. A DNA construct comprising at least one DNA sequence according to Claim 12.

14. A transformed host cell comprising a DNA construct according to Claim 13.

15. The transformed host cell according to Claim 14, wherein said cell is a prokaryote, preferably E. coli or a Streptomycete.

16. The transformed host cell according to Claim 14, wherein said cell is a eukaryote, preferably

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Penicillium chrysogenum, Aspergillus nidulans or Acremonium chrysogenum.

17. Use of a transformed host cell indicated in any one of the Claims 14-16 in obtaining or enhancing the production of β-lactam compounds.

18. A method for obtaining or enhancing production of β-lactam compounds in a microbial host comprising:
    growing a transformed host cell according to any one of the Claims 14-16, or a transformed host cell comprising DNA sequences encoding first and second components or functional portions thereof of a thioredoxin system, each DNA sequence under the regulatory control of transcriptional and translational regulatory signal functional in said cell;
    identifying clones of said transformants which produce said β-lactam compounds at a higher level than said microbial host; and
    isolating said β-lactam compounds.

19. The method according to Claim 18, wherein said microbial host is a fungus, preferably Penicillium chrysogenum, Aspergillus nidulans or Acremonium chrysogenum, or a Streptomyces.

20. A process for reducing disulphide compounds and compounds involved in the production of β-lactam compounds in vitro comprising:
    contacting, in the presence of a reduced electron carrier, the compounds to be reduced with a composition comprising an oxido reductase system, consisting of a HMW polypeptide and a LMW polypeptide, and being capable of reducing disulphide compounds, or with a LMW polypeptide in the presence of a reducing agent as for instance DTT.

21. The method according to Claim 20 wherein ACV is reduced in vitro.

22. Use of the oxido reductase system obtainable from P. chrysogenum as a reducing system in vitro.

19

Figure 1

```
1           10          20          30          40          50          60
GCCTCGGTCTTCTCCAAGTCATTTCCGAGCGTCGCGCGTCAATTCCAATACCGCCAGAAT
                                                            Me

        70          80          90         100         110         120
GGTGCACTCTAAAGTAGTCAgtaagtggttttttcgaggagatcgaatgatccggatcacc
tValHisSerLysValValI

       130         140         150         160         170         180
tagatttcttcgatatcagagttctaaccgttttcccccacagTTATTGGCTCCGGCCCT
                                           leIleGlySerGlyPro

       190         200         210         220         230         240
GGTGCCCACACTGCCGCTATCTATCTATCGCGCGCAGAGCTCCAGCCAGTCCTTTACGAG
GlyAlaHisThrAlaAlaIleTyrLeuSerArgAlaGluLeuGlnProValLeuTyrGlu

       250
GGCATGCTCG
GlyMetLeu
```
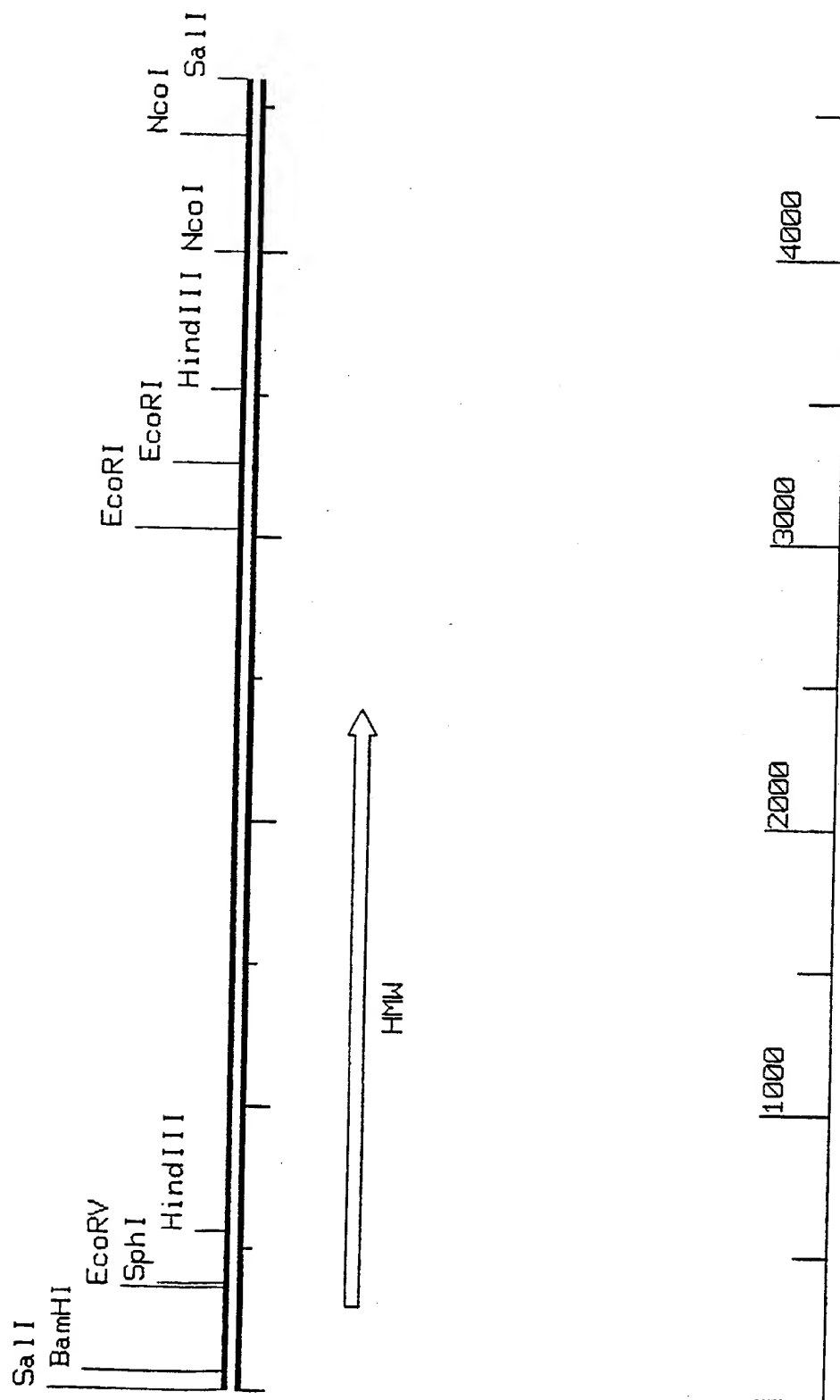
Figure 2

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	ANNUAL REVIEW OF BIOCHEMISTRY vol. 54, 1985, ANNUAL REVIEWS INC. pages 237 - 271; HOLMGREN ARNE; 'Thioredoxin ' * the whole document *	1-22	C12N9/02 C12N15/53 C12P35/00
O,A	EP-A-354 624 (GIST-BROCADES N.V.) * the whole document *	1-22	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C12N
C12P
C07K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	17 SEPTEMBER 1991	ANDRES S.M.

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